Molecular Characterization and Phylogenetic Analysis of *Pseudomonas aeruginosa* Obtained from Wound Infection

Shobha Mehra[1], Charu Tyagi[2], Namrata Dudha[1], Yugandhar P. Reddy[3], Jayanand Manjhi[1], Varun Kumar Sharma[1*] and Lomas Kumar Tomar[1]

[1]Department of Biotechnology & Microbiology, School of Sciences, Noida International University-NIU, Gautam Budh Nagar-201308, Uttar Pradesh, India.

[2]Department of Biotechnology, VSPG (PG) College, CCS University, Meerut-250004, Uttar Pradesh, India.

[3]Department of Zoology, Government Degree College, Pattikonda, Kurnool- 518380, Andhra Pradesh, India.

*E-mail: varungenetics@gmail.com

Abstract

Purpose: Wounds infection is frequent hospital acquired infections which is caused by a wide variety of microorganism. Inappropriate treatment could be more hazardous to suffering patients and could lead to death. This manuscript is aimed to identify the existence of *Pseudomonas aeruginosa* from the clinical samples and determine the molecular evolution and population structure of *Pseudomonas* species using bioinformatics tools

Method: The specimens were swiped from the bandage of infected patients, who were admitted for the therapeutic intervention in the hospitals, localized in Delhi-NCR region. Primarly, specimens were cultured in cetrimide broth for specific growth of *Pseudomonas sp*. Single colony isolation on cetrimide agar plate and characterization of bacterial colony by producing fluorescence under UV light was done to confirm *Pseudomonas aeruginosa (P. aeruginosa)*. Furthermore, phylogenetic analysis was performed on the basis of PCR and sequencing of 16s rRNA sequence of *P. aeruginosa*.

Results: Assay based on bacterial culture, biochemical and 16S rRNA gene analysis methods confirm the presence of *P. aeruginosa* in collected wound specimen. Basic local alignment search tool analysis indicates that newly sequenced 16S rRNA gene sequence was shown 99% similarity to *P. aeruginosa* species, analyzed using NCBI-BLAST tool. The phylogenetic analysis and nucleotide base composition studies performed using 45 sequences of 16S rRNA gene from 18 different species of *Pseudomonas*, including *P. aeruginosa*. The phylogenetic analysis was performed using Maximum Likelihood method for evolutionary relationships.

Conclusion: The biochemical and molecular characterization revealed that the clinical samples were infected with *P. aeruginosa*. Further, phylogenetic analysis of 16S RNA sequences revealed evolutionary conservation among different *Pseudomonas* species.

Keywords: 16s RNA; maximum likelihood; *pseudomonas* species; phylogenetic analysis; wound infection

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Introduction

Wounds bacterial contamination are the common hospital acquired infections causing more than 80% of the mortality (Manikandan et al., 2013). Wounds infection have been recognized as the most critical problem especially in the presence of foreign materials that increases the risk of serious infection even with relatively small bacterial infection (Rubin RH 2006). Nosocomial infection is usually higher in burn patients that correlates with other factors like nature of burn injury, extent of injury, age of patient and burn depth. Other microbial factors such as type, number of organisms, colonization of the burn wound site, enzymes, toxins production, systemic dissemination of the colonizing organisms, have a strong effect on severity of bacterial wound infection (Pruitt et al., 1998). In addition, widespread inappropriate non-judicious use of antibiotics has resulted in a significant development of antibiotic resistance in wound infecting bacteria (Sani et al., 2012) that subsequently increases the complications and costs of treatment (Anguzu et al., 2007).

The most common bacterial genera infecting wounds are *Enterococci, Escherichia, Klebsiella, Enterobacter, Proteus, Acinetobacter* and *Pseudomonas* (Gautam et al., 2013). Out of these *Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium that has been known to cause nosocomial infection and thus complicate health care facilities (Kipnis et al., 2006). It accounts for around 30-40% of opportunistic infections in hospitals across globe and is one of the causative agents of hospital acquired pneumonia, (Driscoll et al., 2007). Due to the extended spread of *P. aeruginosa* habitat, the control of the organism in a hospital setting is very difficult and makes it practically impossible to prevent contamination (Davies J 1994). The major threat is the infection of patients who are immunocompromised or those in burns, neonatal and cancer wards (Khalil et al., 2015). Infection of P. aeruginosa is still one of the main causes of death among the critically ill and patients with impaired immune systems despite the development of newer and stronger antibiotics. The genus *Pseudomonas* contains more than 145 species, most of which are saprophytic. More than 27 species are associated with humans. Most *pseudomonads* known to cause disease in humans are associated with pathogenic infections. These include *P. aeruginosa, P. fluorescens, P. cepacia, P. putida, P. stutzeri, P. putrefaciens* and *P. maltophilia*. Only two species, *P. pseudomallei* and *P. mallei produce* specific human diseases: glanders and melioidosis. *Pseudomonas aeruginosa, P. putrefaciens* and *P. maltophilia* account for approximately 80 percent of pseudomonads recovered from clinical specimens.

The genomes of *P. aeruginosa* strains are larger than those of most sequenced bacteria, varying from 5.2 to 7.1 Mbp (Schmidt et al., 1996). The divergence in genome size is caused by the so-called accessory genome. The core genome, with a few exceptions of loci that are subject to diversifying selection, is highly conserved among clonal complexes and shows sequence diversities of 0.5–0.7% (Cramer et al., 2011). The elements of the accessory genome have apparently been acquired by horizontal gene transfer from different sources, including other species. Therefore, a *Pseudomonas aeruginosa* chromosome is often described as a mosaic structure of a conserved core genome frequently interrupted by the inserted portions of the accessory genome. The individual mosaics also show remarkable plasticity (Klockgether et al., 2011). The ongoing acquisition of new foreign DNA, larger or smaller deletion events, the mobilization of prophages, mutations of single nucleotides and even chromosomal inversions, are potentially affecting portions of the core and the accessory genome and

these processes continuously modify the genome and modulate the phenotype of a *Pseudomonas aeruginosa* strain, thus differentiating the strains from each other.

Materials and Methods

Patient specimen collection

The study was carried out over a 4-months period at the hospitals in Delhi-NCR region of India. A total of 497 number of specimens were obtained from burn patients suffering with wound infection. Samples were collected during the removal of bandages and by swabbing the wound after appropriate consent from the patients.

Qualitative conventional detection

Specimen samples were inoculated primarily onto several media such as blood agar, MacConkey agar and cetrimide agar for general microbial culture, and incubated then at 37ºC for 48 h (King EO et al., 2005). The isolates were presumptively identified by routine tests, including colony morphology and pigment formation on selective medium, positive oxidase test, glucose fermentation, hydrolysis of gelatin and growth at 42ºC. (Masuda N et al., 1995).

Bacterial DNA isolation Characterization of bacterial species

Well established culture of bacteria from cetrimide agar were further collected and processed for DNA isolation by CTAB method (Minas K et al., 2011). Briefly, Bacterial colony were broken by grinding with glass rods and added CTAB extraction buffer (Tris Buffer100mM; 25mM EDTA; 1.5M NaCl; 2% CTAB; pH 8.0) incubated at 65°C for 20 min, followed by purification with phenol: chloroform: isoamyl alcohol (25 : 24 : 1) and precipitation with isopropanol were conducted. Precipitated DNA were washed with 70% ethanol. Air dried DNA were re-suspended in 50 µl TE buffer (10 mM Tris buffer; 1 mM EDTA; pH 8.0) and stored at -200 C.

Amplification of 16S rRNA gene using PCR

For the PCR reaction, total reaction volume was 50 µl, containing 5 µl of purified template DNA, 2U of DNA polymerase, 10 pmol of each primer (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer: 5'-CTTGTGCGGGCCCCCGTCAATTC-3') 200 µmol of each deoxyribonucleoside t riphosphate per liter, 1.5 mmol of MgCl2 per liter, 10 mmol of Tris-HCl (pH 8.8) per liter, 50 mmol of KCl per liter, and 0.1% Triton X-100.

Amplification of 16S rRNA gene was performed in a PCR Thermo Cyclers (MJ Research PTC 200) for 35 cycles by using the following parameters: denaturation at 95°C for 30 second, annealing at 60°C for 40 second, and extension at 72°C for 1 min. The cycles were preceded by a denaturation step at 95°C for 3 min, followed by an extension step at 72°C for 7 min.

Sequencing analysis

The PCR product was purified using QIAquick gel extraction kit (Qiagen, Germany). 15µl of the purified product was sequenced by use of the ABI Prism DNA sequencing kit, Big Dye Terminator Cycle Sequencing (version 2.0 or 3.0), and ABI Prism 310 genetic analyzer (Applied Biosystems, USA).

Sequence retrieval

16S ribosomal RNA gene sequences of different species of *Pseudomonas* were retrieved from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) databases in FASTA format, listed in Table 1.

Local sequence alignment

Sequence alignment of the sample test 16S rRNA sequence NDVKS1 and sequences retrieved from NCBI was carried out using NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) suit of tools to identify the homology or similarity in different species of *Pseudomonas*

Nucleotide base composition of the 16S rRNA gene sequence of *Pseudomonas* species

Sequences were analyzed to determine the diversity in respect to percentage of Adenine, Thymine, Guanine and Cytosine, AT content, GC content, relative melting temperature of 16S ribosomal RNA gene sequence of different species of *Pseudomonas* using MEGA 7 (Kumar et al., 2016) software. For nucleotide base composition analysis, we have covered 926bp length of 16S rRNA gene sequence of 18 different species of *Pseudomonas* such as, P. aeruginosa, P. agarici, P. alcaligenes, P. alcaliphila, P. chlororaphis, P. citronellolis, P. fulva, P. jinjuensis, P. marginalis, P. nitroreducens, P. oryzihabitans, P. parafulva, P. pseudoalcaligenes, P. putida, P. resinovorans, P. rhodesiae, P. straminea and P. stutzeri.

Phylogenetic analysis

Phylogenetic analysis of *Pseudomonas* 16S ribosomal RNA gene sequence through Maximum likelihood methods were carried out using MEGA7 software (Kumar et al., 2016). Phylogenetic trees were constructed by the software showing the ancestral relationship among the sequences. The Maximum Likelihood phylogenic tree give different clusters showing their evolution relationship with each other and tree reveals different clade showing their evolutionary relationship within different species of *Pseudomonas*. The sequences which lie in the same cluster are closely related.

Results and Discussion

Sequence retrieval

Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence was retrieved from the NCBI in FASTA format. Figure 1 shows the sequence of the gene (NR_117678.1).

Fig 1. FASTA format of reference sequence of *Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA gene indicating the forward primer in Red and reverese primer in blue colour. The given primers were used to sequence 16S rRNA of the isolate NDVKS1.

>NR_117678.1 *Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA gene, partial sequence

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
GGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
GCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGA
GAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
CACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACA
GAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTG
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
ACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAG
TGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGG
GAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAG
GAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTA
CGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTG
GAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAA
GTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAA
GGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTA
GGGGAACCTGCGGCTGGATCACCTCCTTT

Local sequence alignment

The newly *Pseudomonas aeruginosa* strain 16S ribosomal RNA gene NDVKS1 (Submission ID-2577877), sequence was used to perform BLAST analysis using NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). After performing BLAST, the NCBI BLAST tool produced BLAST table (list of the aligned sequence) showing the accession numbers, percent similarity, e-value, etc (Table 1). The sequences having lowest e-value were more closely related while the difference in e-value shows the dissimilarity among them. All high degree of homologous sequences of *Pseudomonas* were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/nuccore) in FASTA format.

Table 1. BLAST table of 16s RNA of P. aeruginosa strain

S. No.	ACCESSION	E VALUE	ORGANISM	DESCRIPTION	% IDENTITY
1	NDVKS1	6.00E-82	*P. aeruginosa*	*P. aeruginosa strain NDVKS1 16S ribosomal RNA gene, partial sequence*	100%
2	NR_117678.1	6.00E-82	*Pseudomonas Spps*	*P. aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence*	99%
3	NR_113599.1	6.00E-82	*Pseudomonas Spps*	*P. aeruginosa strain NBRC 12689 16S ribosomal RNA gene, partial sequence*	99%
4	NR_114471.1	6.00E-82	*Pseudomonas Spps*	*P. aeruginosa strain ATCC 10145 16S ribosomal RNA gene, partial sequence*	99%

5	NR_026078.1	1.00E-78	*Pseudomonas Spps*	*P. aeruginosa strain DSM 50071 16S ribosomal RNA, complete sequence*	99%
6	NR_149822.1	1.00E-73	*Pseudomonas Spps*	*P. sesami strain SI-P133 16S ribosomal RNA, partial sequence*	97%
7	NR_116905.1	3.00E-70	*Pseudomonas Spps*	*P. saponiphila strain DSM 9751 16S ribosomal RNA, partial sequence*	96%
8	NR_043730.1	3.00E-70	*Pseudomonas Spps*	*P. pohangensis strain H3-R18 16S ribosomal RNA gene, partial sequence*	96%
9	NR_114749.1	3.00E-70	*Pseudomonas Spps*	*P. protegens strain CHA0 16S ribosomal RNA, partial sequence*	96%
10	NR_145562.1	3.00E-70	*Pseudomonas Spps*	*P. glareae strain KMM 9500 16S ribosomal RNA, partial sequence*	95%
11	NR_135703.1	2.00E-67	*Pseudomonas Spps*	*P. guariconensis strain PCAVU11 16S ribosomal RNA, partial sequence*	95%
12	NR_044209.1	7.00E-67	*Pseudomonas Spps*	*P. panipatensis strain Esp-1 16S ribosomal RNA gene, partial sequence*	94%
13	NR_043174.1	7.00E-67	*Pseudomonas Spps*	*P. segetis strain FR1439 16S ribosomal RNA gene, partial sequence*	94%
14	NR_112062.1	7.00E-67	*Pseudomonas Spps*	*P. resinovorans strain ATCC 14235 16S ribosomal RNA, partial sequence*	94%
15	NR_026534.1	7.00E-67	*Pseudomonas Spps*	*P. resinovorans strain LMG 2274 16S ribosomal RNA gene, partial sequence*	94%
16	NR_114957.1	8.00E-66	*Pseudomonas Spps*	*P. guezennei strain RA26 16S ribosomal RNA, partial sequence*	94%
17	NR_043289.1	8.00E-66	*Pseudomonas Spps*	*P. otitidis strain MCC10330 16S ribosomal RNA, partial sequence*	94%
18	NR_114194.1	3.00E-65	*Pseudomonas Spps*	*P. citronellolis strain NBRC 103043 16S ribosomal RNA gene, partial sequence*	94%
19	NR_116904.1	3.00E-65	*Pseudomonas Spps*	*P. benzenivorans strain DSM 8628 16S ribosomal RNA, partial sequence*	94%
20	NR_041036.1	3.00E-65	*Azomonas Spps*	*Azomonas macrocytogenes strain IAM 15003 16S ribosomal RNA gene, partial sequence*	94%
21	NR_041247.1	3.00E-65	*Pseudomonas Spps*	*P. azotifigens strain 6H33b 16S ribosomal RNA, partial sequence*	94%
22	NR_112069.1	3.00E-65	*Pseudomonas Spps*	*P. citronellolis strain ATCC 13674 16S ribosomal RNA, partial sequence*	94%
23	NR_041952.1	3.00E-65	*Pseudomonas Spps*	*P. abietaniphila strain BKME-9 16S ribosomal RNA gene, partial sequence*	94%
24	NR_026533.1	3.00E-65	*Pseudomonas Spps*	*P. citronellolis strain DSM 50332 16S ribosomal RNA gene, partial sequence*	94%

25	NR_114196.1	3.00E-65	*Pseudomonas Spps*	*P. indica strain NBRC 103045 16S ribosomal RNA gene, partial sequence*	93%
26	NR_028801.1	3.00E-65	*Pseudomonas Spps*	*P. indica strain IMT37 16S ribosomal RNA gene, partial sequence*	93%
27	NR_103934.2	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain ATCC 17588 16S ribosomal RNA, complete sequence*	93%
28	NR_114215.1	1.00E-63	*Pseudomonas Spps*	*P. luteola strain NBRC 103146 16S ribosomal RNA gene, partial sequence*	93%
29	NR_113652.1	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain NBRC 14165 16S ribosomal RNA gene, partial sequence*	93%
30	NR_109470.1	1.00E-63	*Pseudomonas Spps*	*P. zeshuii strain BY-1 16S ribosomal RNA gene, partial sequence*	93%
31	NR_116489.1	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain VKM B-975 16S ribosomal RNA gene, partial sequence*	93%
32	NR_043731.1	1.00E-63	*Pseudomonas Spps*	*P. delhiensis strain RLD-1 16S ribosomal RNA gene, partial sequence*	93%
33	NR_041715.1	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain ATCC 17588 16S ribosomal RNA gene, partial sequence*	93%
34	NR_114751.1	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain DSM 5190 16S ribosomal RNA gene, partial sequence*	93%
35	NR_037134.1	1.00E-63	*Pseudomonas Spps*	*P. luteola strain 4239 16S ribosomal RNA gene, partial sequence*	93%
36	NR_118798.1	1.00E-63	*Pseudomonas Spps*	*P. stutzeri strain CCUG 11256 16S ribosomal RNA, partial sequence*	93%
37	NR_117186.1	2.00E-62	*Pseudomonas Spps*	*P. marincola strain AB251f 16S ribosomal RNA gene, partial sequence*	93%
38	NR_156987.1	7.00E-62	*Pseudomonas Spps*	*P. paralactis strain DSM 29164 16S ribosomal RNA, partial sequence*	93%
39	NR_134793.1	7.00E-62	*Pseudomonas Spps*	*P. matsuisoli strain CC-MHH0089 16S ribosomal RNA, partial sequence*	93%
40	NR_114481.1	7.00E-62	*Pseudomonas Spps*	*P. tolaasii strain ATCC 33618 16S ribosomal RNA gene, partial sequence*	93%
41	NR_126210.1	7.00E-62	*Pseudomonas Spps*	*P. aestusnigri strain VGXO14 16S ribosomal RNA gene, partial sequence*	93%
42	NR_114227.1	7.00E-62	*Pseudomonas Spps*	*P. tolaasii strain NBRC 103163 16S ribosomal RNA gene, partial sequence*	93%

43	NR_114225.1	7.00E-62	*Pseudomonas Spps*	*P. mucidolens strain NBRC 103159 16S ribosomal RNA gene, partial sequence*	93%
44	NR_113583.1	7.00E-62	*Pseudomonas Spps*	*P. synxantha strain NBRC 3913 16S ribosomal RNA gene, partial sequence*	93%
45	NR_042199.1	7.00E-62	*Pseudomonas Spps*	*P. lurida strain P 513/18 16S ribosomal RNA gene, partial sequence*	93%
46	NR_117823.1	7.00E-62	*Pseudomonas Spps*	*P. tolaasii 16S ribosomal RNA gene, partial sequence*	93%
47	NR_028987.1	7.00E-62	*Pseudomonas Spps*	*P. trivialis strain P 513/19 16S ribosomal RNA gene, partial sequence*	93%
48	NR_028986.1	7.00E-62	*Pseudomonas Spps*	*P. poae strain P 527/13 16S ribosomal RNA gene, partial sequence*	93%
49	NR_041592.1	7.00E-62	*Pseudomonas Spps*	*P. marincola strain KMM 3042 16S ribosomal RNA gene, partial sequence*	93%
50	NR_043935.1	7.00E-62	*Pseudomonas Spps*	*P. chlororaphis subsp. aurantiaca strain NCIB 10068 16S RNA gene, partial sequence*	93%

It is clear from the BLAST results that P. aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence (NR_117678.1), complete sequence (NR_026078.1), P. aeruginosa strain NBRC 12689 16S ribosomal RNA gene, partial sequence (NR_113599.1), P. aeruginosa strain ATCC 10145 16S ribosomal RNA gene, partial sequence (NR_114471.1), are P. aeruginosa strain 16S ribosomal RNA genes which were 99% identical with newly sequence P. aeruginosa strain 16S ribosomal RNA genes. Additionally, other *Pseudomonas* species; P. sesame, P. saponiphila, P. pohangensis, P. protegens, P. glareae and P. guariconensis are also 95-97% identical with new sequenced P. aeruginosa strain 16S ribosomal RNA genes sequence., while other species of *Pseudomonas*: P. panipatensis, P. segetis, P. resinovorans, P. guezennei, P. otitidis, P. citronellolis, P. benzenivorans, P. azotifigens, P. abietaniphila, P. indica, P. stutzeri, P. luteola, P. zeshuii, P. delhiensis, P. marincola, P. paralactis, P. matsuisoli, P. tolaasii, P. aestusnigri, P. mucidolens, P. synxantha, P. lurida, P. trivialis, P. poae, P. chlororaphis strain 16S ribosomal RNA genes were the most dissimilar sequences with 93 % identity (Table 1).

Nucleotide base composition (mol %) of the 16S ribosomal RNA gene sequence of *Pseudomonas*

The nucleotide base composition (mol %) of the sequences of P. aeruginosa, P. agarici, P. alcaligenes, P. alcaliphila, P. chlororaphis, P. citronellolis, P. fulva, P. jinjuensis, P. marginalis, P. nitroreducens, P. oryzihabitans, P. parafulva, P. pseudoalcaligenes, P. putida, P. resinovorans, P. rhodesiae, P. straminea and P. stutzeri have been depicted in Table 2.

Previous studies on based on other microorganisms indicate that there is a direct relation between the molecular composition of nucleotides (primarily GC content) and growth temperatures (Topt)of microbes (Rudi, 2009; Hu et al., 2022). Moreover, it has been previously established the structural RNAs are more sensitive to temperature variation

in comparison to genomic DNA (Hu et al., 2022). Therefore, the molecular composition (GC and AT content) in *Pseudomonas* species was analyzed to evaluate the role of GC content with respect to the survival of the bacterium. GC and AT content of P. aeruginosa ranged from 54.3 to 52.0 and 45.7 to 45.6, 54.0 to 53.7 and 46.0 to 46.4 respectively. In P. agarici. In P. alcaligenes and P. alcaliphila GC and AT content varied from 53.8 to 53.6 and 46.2 to 46.4, 53.9 to 53.9 and 46.1 to 46.1 respectively. GC and AT content of P. chlororaphis, P. citronellolis ranged from 54.1 to 54.0 and 45.9 to 46.0, 54.5 to 54.4 and 45.6 to 45.6 respectively. In P. fulva, P. jinjuensis GC and AT content varied from 53.7 to 53.6 and 46.3 to 46.4, 54.4 to 54.3 and 45.6 to 45.7 respectively. GC and AT content of P. marginalis, P. nitroreducens, P. oryzihabitans, P. parafulva, P. pseudoalcaligenes, P. putida, P. resinovorans, P. rhodesiae, P. straminea and P. stutzeri ranged from 54.4,45.6 to 54.3,45.7, 53.9,46.1 to 53.7,46.3, 53.6, 46.4 to 53.7,46.3, 54.1,45.9 to 54.0,46.1, 53.9, 46.2.6 to 53.6, 46.4, 54.3, 45.7 to 54.6, 45.3, 53.7, 46.3 to 53.9, 46.2, 53.5, 46.5 to 53.4, 46.6 and 53.8, 46.2 to 53.6, 46.4 respectively.

The comparative analysis of nucleotide content among different *Pseudomonas* species revealed no substantial variation in the GC content. Hence, we assumed that no significant change occurred in the Topt of the selected species. Thus, indicating that there is no change in the structural composition of the rRNA.

Table 2. Nucleotide base composition (mol %) of the 16S ribosomal RNA gene sequence of *Pseudomonas* species

Accession no.	***Pseudomonas Spps***	Adenine (mol%)	Guanine (mol%)	Cytosine (mol%)	Thymine (mol%)
M34133.1	*Pseudomonas aeruginosa*	25.5	31.8	22.6	20.1
NR 113599.1	*Pseudomonas aeruginosa*	25.4	31.7	22.6	20.3
NR 114471.1	*Pseudomonas aeruginosa*	25.4	31.6	22.6	20.3
NR 117678.1	*Pseudomonas aeruginosa*	25.2	31.6	22.7	20.4
NR 036998.1	*Pseudomonas agarici*	25.6	31.5	22.5	20.4
NR 115608.1	*Pseudomonas agarici*	25.7	31.4	22.3	20.7
NR 113646.1	*Pseudomonas alcaligenes*	25.5	31.3	22.5	20.7
NR 114472.1	*Pseudomonas alcaligenes*	25.5	31.1	22.6	20.7
NR 117827.1	*Pseudomonas alcaligenes*	25.6	31.2	22.4	20.8
NR 024734.1	*Pseudomonas alcaliphila*	25.3	31.5	22.4	20.8
NR 114072.1	*Pseudomonas alcaliphila*	25.2	31.5	22.4	20.9
NR 044974.1	*Pseudomonas chlororaphis*	25.3	31.6	22.5	20.6
NR 113581.1	*Pseudomonas chlororaphis*	25.2	31.6	22.4	20.7
NR 114474.1	*Pseudomonas chlororaphis*	25.2	31.4	22.6	20.8
NR 026533.1	*Pseudomonas citronellolis*	25	31.9	22.4	20.7
NR 112069.1	*Pseudomonas citronellolis*	25.2	32	22.5	20.4
NR 114194.1	*Pseudomonas citronellolis*	25.2	32	22.4	20.4
NR 104280.1	*Pseudomonas fulva*	25.4	31.3	22.3	21
NR 113857.1	*Pseudomonas fulva*	25.3	31.4	22.3	21
NR 115610.1	*Pseudomonas fulva*	25.2	31.2	22.4	21.2
NR 025226.1	*Pseudomonas jinjuensis*	25.2	31.8	22.6	20.4
NR 114197.1	*Pseudomonas jinjuensis*	25.3	31.8	22.5	20.4
NR 112072.1	*Pseudomonas marginalis*	25.2	31.6	22.3	20.9

NR 117821.1	*Pseudomonas marginalis*	25.4	31.3	22	21.3
NR 042435.1	*Pseudomonas nitroreducens*	25.1	31.7	22.7	20.5
NR 113601.1	*Pseudomonas nitroreducens*	25.2	31.8	22.5	20.5
NR 114041.1	*Pseudomonas oryzihabitans*	25.5	31.3	22.6	20.6
NR 115005.1	*Pseudomonas oryzihabitans*	25.6	31.2	22.5	20.7
NR 040859.1	*Pseudomonas parafulva*	25.3	31.3	22.3	21.1
NR 113856.1	*Pseudomonas parafulva*	25.3	31.4	22.3	21
NR 037000.1	*Pseudomonas pseudoalcaligenes*	25.3	31.6	22.5	20.6
NR 113653.1	*Pseudomonas pseudoalcaligenes*	25.3	31.6	22.4	20.8
FM211694.1	*Pseudomonas putida*	25.5	31.4	22.5	20.7
NR 113651.1	*Pseudomonas putida*	25.5	31.3	22.3	20.9
NR 026534.1	*Pseudomonas resinovorans*	25.1	31.4	22.9	20.6
NR 112062.1	*Pseudomonas resinovorans*	25.2	31.9	22.7	20.1
NR 024911.1	*Pseudomonas rhodesiae*	25.2	31.4	22.3	21.1
NR 112074.1	*Pseudomonas rhodesiae*	25.2	31.5	22.4	21
NR 036908.1	*Pseudomonas straminea*	25.3	31	22.5	21.2
NR 113859.1	*Pseudomonas straminea*	25.5	31.2	22.2	21.1
NR 113652.1	*Pseudomonas stutzeri*	25.5	31.4	22.4	20.7
NR 114751.1	*Pseudomonas stutzeri*	25.8	31.4	22.1	20.7
NR 116489.1	*Pseudomonas stutzeri*	25.6	31.3	22.3	20.8

Phylogenetic analysis

The phylogenetic analysis included the reference strains of *Pseudomonas* species that were retrieved from NCBI database. *Pseudomonas* species 16S rRNA gene alignments were generated. Individual dendrograms were generated using different methods, namely the maximum likelihood methods. Phylogenetic groups and subgroups were defined by the length and branching order of the concatenated gene tree. The resulting groups were supported by high bootstrap values.

In Phylogenetic analysis, alignment of nucleotide sequences is a major consideration, particularly in studies of genes from divergent taxa. It seems obvious to state that the phylogenetic analysis of sequences begins with the appropriate alignment of the data themselves, yet alignment remains one of the most difficult and poorly understood facets of molecular data analysis. Alignment of the genomic sequences are required to analyze the phylogenetic tree. Phylogenetic analysis often includes the search for evidence of directional selection in molecular evolution (Hsu et al., 2005; Hofmann et al., 2003). Evolution of the 16S RNA was studied in different organisms and adaptive changes were in the sequences. The phylogenetic analysis of the *Pseudomonas* species 16S rRNA gene dataset resulted in a tree consistent with modern systematic understanding of the relatedness among different species, mainly based on DNA sequences homology. This provides strong support for the quality of our sequences and bioinformatics methods. The posterior probability values at the nodes indicate strong support for the branch splits, providing further support for this tree (Figure 1).

In order to determine the genus of the bacterial isolates collected from the patient's specimen, we sequenced the 16S rRNA gene. The obtained sequences were BLAST

against NCBI's 16S rRNA GenBank (Altschul et al., 1990). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-960.3025) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The phylogenetic analysis performed using 45 sequences of 16S rRNA gene from 18 different species of *Pseudomonas*, including P. aeruginosa. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

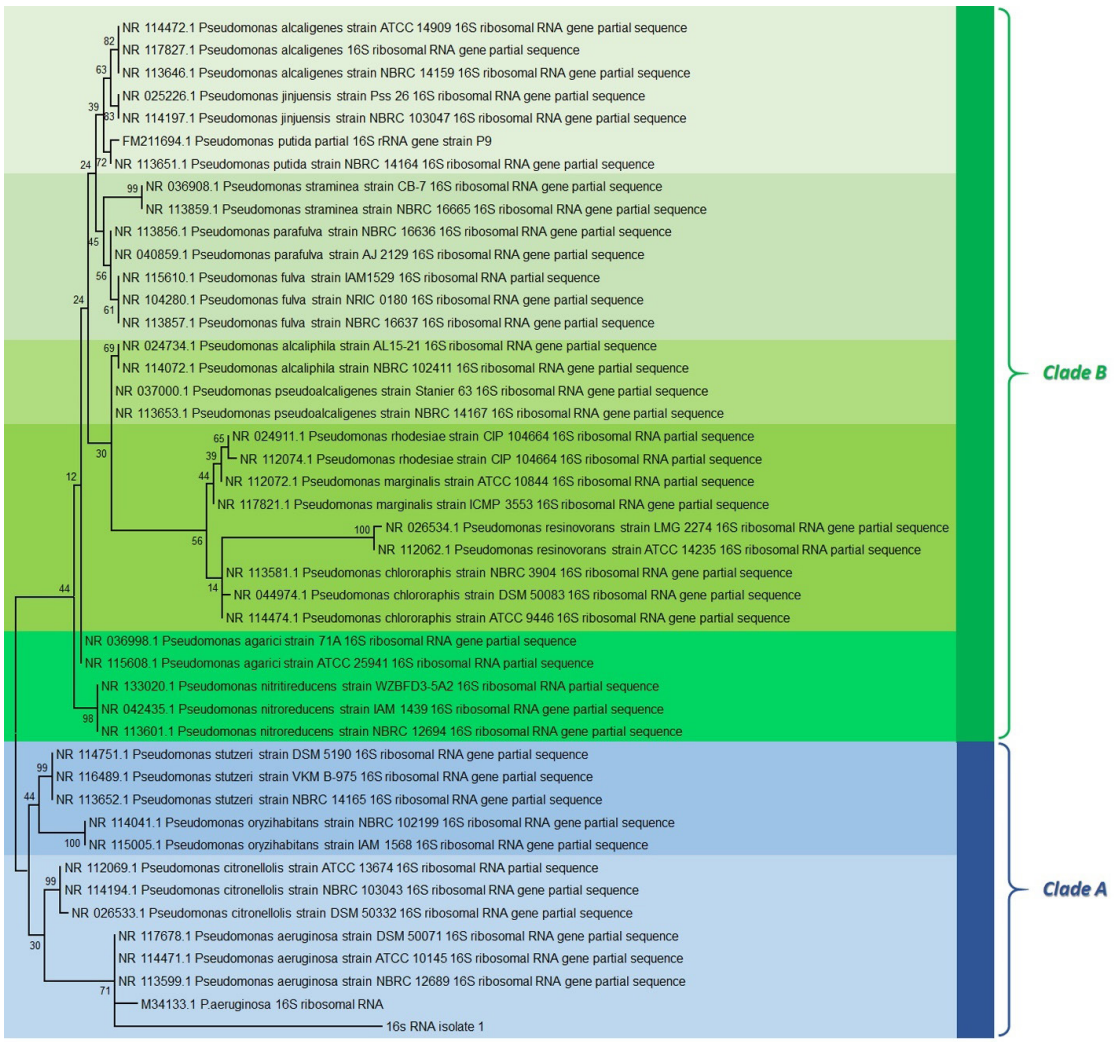

Figure 2. Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-960.3025) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016)

The phylogenetic tree were constructed using Maximum likelihood method for the sequence of newly isolated bacteria. Maximum likelihood method is most suitable model to understand the evolutionary history of an organism. The bootstrap consensus trees inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed. The Maximum likelihood trees were obtained using the Nearest Neighbor-Interchange heuristic algorithm. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). Phylogenetic analyses were conducted in MEGA7 and obtained two major cluster (depicted in Figure 2), classified as Clade A (Blue color) and Clade B (Green Color). Clade A is further classified in two subgroup named A1 and A2. A1 consists of two major branches with two *pseudomonas* strains named P. aerignosa and P. chionocolois. Interestingly, newly sequenced 16S RNA falls in A1 with P. aerignosa which confirm its existence as P. aerignosa strain. As other group A2 is a cluster of two P. oryzihabitans and P. stutzi. Additionally, Clade B is further classified in five subgroup named B1, B2, B3, B4 and B5. B1 consists of two major branches with two *pseudomonas* strains named P. nitroreducens and P. agarici. As other group B2 is a cluster of four P. chlororaphis, P. resinovorans, P. marginalis and P. rhodesiae. B3 consists of two major branches with two *pseudomonas* strains named P. pseudoalcaligenes and P. alcaliphila. As other group B4 and B5 is a cluster of Three P. fulva, P. parafulva, P. straminea. and P. putida, P. jinjuensis, P. alcaligenes.

The observations based on phylogenetic analysis of 16s RNA gene of *Pseudomonas* species using Maximum Likehood method revealed the relationships and percent similarity of 16s RNA gene within *Pseudomonas* species, including P. aeruginosa, P. chionocolois, P. oryzihabitans, P. stutzi, P. nitroreducens, P. agarici, P. chlororaphis, P. resinovorans, P. marginalis, P. rhodesiae, P. pseudoalcaligenes, P. alcaliphila, P. fulva, P. parafulva, P. straminea, P. putida, P. jinjuensis, and P. alcaligenes were studied in detail. Phylogenetic analysis and multiple sequence alignment of the P. aeruginosa strain 16S ribosomal RNA gene, partial sequence through various phylogenetic tree were performed which showed its pattern of variations and relationship among different *Pseudomonas* species.

Conclusion

Phylogenetic analysis of the *Pseudomonas* species, including new isolate from medical specimens revealed that they are the same strain and are affiliated to *Pseudomonas aeruginosa*. In recent years, Next Generation of Sequencing technologies boosted the genome databases and a remarkable increase in the number of sequenced genomes, drafts or complete, are available, but the correct assignation of the sequenced strains to the corresponding species with the accepted taxonomic tools is important before comparative analyses with other genomes can be performed. The need for the whole genome sequences of all the type strains, which are the only species references that are publicly available in culture collections, is evident. In the present study, we have identified and characterized *Pseudomonas aeruginosa* from the clinical specimens, using molecular biology techniques. New 16sRNA sequences of P. aeruginosa isolated were aligned with *Pseudomonas* species and constructed phylogeny tree to determine the molecular evolution and population structure of *Pseudomonas* species using bioinformatics tools. The phylogenetic affiliations of the different species of the genus *Pseudomonas* were shown by the Maximum likelihood based phylogenetic analyses using the 16S rRNA sequences. Our study demonstrated that positive selection of 16s RNA gene during the divergence of different species of *Pseudomonas* during evolution.

These evolutionary acquirements have made necessary changes in the genetic control of ontogeny, and this, in turn, might have caused adaptive changes in the 16s RNA gene.

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Conflicts of Interest Statement

The authors declare no conflict of interests.

Ethics Declaration

Ethical clearance (Ref: NIU/NERB/SOS/BT-MB/18/105) was obtained from Institutional Ethical Committee (Ethics Review Board), Noida International University, Gautam Budh Nagar, UP, India. Informed consents were collected from patients whose samples were used for the current study.

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التوصيف الجزيئي والتحليل الوراثي لالزائفة الزنجارية المتحصل عليها من عدوى الجروح

شبحة ميهرا[1]، شارو تياجي[2]، نمراتا دودة[1]، يوجاندهار ب. ريدي[3]، جاياناند مانجي[1]، فارون كومار شارما[1*] ولوماس كومار تومار[1]

[1] قسم التكنولوجيا الحيوية والأحياء الدقيقة ، كلية العلوم، جامعة نويدا الدولية، جامعة نويدا الدولية، غوتام بود ناجار 201308، أوتار براديش، الهند.

[2] قسم التكنولوجيا الحيوية، كلية (VSPG PG)، جامعة Meerut-250004 ، CCS، أوتار براديش، الهند.

[3] قسم علم الحيوان، الكلية الحكومية، باتيكوندا، كورنول - 518380، أندرا براديش، الهند.

[*] البريد الإلكتروني: varungenetics@gmail.com

المُستَخلَص

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الغرض: عدوي الجروح هي عدوي متكررة مكتسبه من المستشفيات وتسببها مجموعه متنوعة من الكائنات الحية الدقيقة. وقد يكون العلاج الغير مناسب أكثر خطورة علي المرضي الدين يعانون ويمكن ان يؤدي إلى الوفاة. تهدف هذه المخطوطة إلى التعرف على وجود *Pseudomonas aeruginosa* من العينات السريرية وتحديد التطور الجزيئي وتركيب البنيه السكانية لأنواع الـ *pseudomonas* باستخدام ادوات المعلوماتية الحيوية.

الطريقة: تم سحب العينات من ضمادة المرضي المصابين الذين تم ادخالهم للتدخل العلاجي في المستشفيات المتمركزين في منطقه دلهي ncr في البداية تم استزراع العينات في بيئة سترميد لنمو معين من *Pseudomonas* sb. تم عزل مستعمره مفرده علي طبق آجار سترميد وتوصيف المستعمرة البكتيرية عن طريق إنتاج وميضان تحت ضوء الأشعة فوق البنفسجية لتأكيد *Pseudomonas aeruginosa* (*P. aeruginosa*) علاوة علي ذلك تم إجراء تحليل التطور الجيني علي أساس تفاعل البلوميريز المتسلسل وتتابع الـ ر ن ا الريبوزي 16S من الـ *P. aeruginosa*. النتائج الفحص علي أساس المستعمرة البكتيرية والطرق البيوكيميائية وطرق التحليل الجيني 16S rRNA تؤكد وجود ال *P. aeruginosa* التي جمعت من عينات الجروح.

النتائج: يشير تحليل أداة بحث المحاذاة المحلية الأساسية الي ان تسلسل الجين 16S rRNA المتسلسل حديثا قد اضهر تشابها بنسبة 99% مع انواع ال *P. aeruginosa* وتم تحليله باستخدام اداة NCBI-BLAST.تم اجراء دراسات التحليل الوراثي والتكوين الاساسي للنيكيولوتيدات باستخدام 45 تتابعات من جين 16S rRNAمن 18 نوعا مختلفا من *Pseudomonas* بما في ذلك ال *P. aeruginosa*. تم اجراء تحليل التطور الجيني باستخدام طريقة الاحتمالية القصوى للعلاقات التطورية .

الخلاصة: اظهر التوصيف البيوكيميائي والجزيئي علي ان العينات السريرية مصابه ببكتيريا الـ *aeruginosa*. P علاوة علي ذلك كشف التحليل الوراثي لتتابع 16S rRNA عن الحفظ التطوري بين انواع الـ *Pseudomonas* المختلفة.

الكلمات المفتاحية: 16s RNA؛ أقصى احتمال أنواع الزائفة؛ تحليل النشوء والتطور؛ عدوى الجرح.